Formulation Effects on Immune Response to Nanocarriers Encapsulating TLR 7 Agonist

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Dedication

This thesis is dedicated to my supportive and excellent parents – Lijuan Liu and Quanguo Wang.

Abstract

Recent decades have witnessed remarkable progress in cancer immunotherapy as an approach to enhancing host immune response against cancer. Particularly, a cancer vaccine comprised of antigen, vaccine adjuvant, and delivery system has gained widespread attention, which can elicit immune response by activating dendritic cells (DCs), the critical antigen-presenting cells (APCs) [1].

In the past decades, nanoformulations have gained extensive attention as drug carriers for improved cancer immunotherapy. Imidazoquinoline-based toll-like receptor (TLR) 7 agonist, imiquimod (IMQ), a cytokine inducer, could elicit DC activation. TLR7 activation stimulates myeloid differentiation primary-response gene 88 (MyD88) signaling pathways, elicit DCs to upregulate costimulatory molecules, secrete type I interferons and pro-inflammatory cytokines, and stimulate T cell-mediated immune response [2].

The development of a variety of nanoformulations as drug carriers, such as polymeric poly(lactide-co-glycolide) (PLGA) nanoparticles (NPs) and liposomes, has broadened the application of TLR7 agonist in cancer immunotherapy. However, an improved understanding of how formulation factors could influence the immune response to nanocarriers encapsulating TLR 7 agonist can drive the discovery of more efficient platforms to deliver TLR 7 agonist to immune system for enhanced cancer immunotherapy. In this thesis, we encapsulated IMQ in PLGA NPs that were either naturally anionic or modified with didodecyldimethylammonium bromide (DMAB) to generate cationic surface charge. In addition, 18:0 PC 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) were employed to formulate IMQ-loaded anionic DSPC liposomes and cationic DOTAP liposomes. These formulations were evaluated for *in vitro* DC activation and antigen presentation with a model antigen, ovalbumin (OVA), using bone marrow-derived dendritic cells (BMDCs) and DC 2.4 cell line. Cell viability assay showed that PLGA NPs and DSPC liposomes showed negligible cytotoxicity on BMDCs and DC 2.4 cells at low concentrations, whereas DMAB-PLGA NPs and

DOTAP liposomes exhibited obvious cytotoxicity at relatively low concentrations. Also, anionic PLGA NPs were superior to other nanoformulations in eliciting costimulatory molecule expression by DCs, whereas cationic DOTAP liposomes were superior in inducing antigen presentation by DCs compared with other nanoformulations.

Overall, our studies demonstrated that IMQ loaded PLGA NPs showed both better biocompatibility and stronger DC activation efficacy compared with other formulations. However, further studies are needed to understand the mechanism of formulation effects on immune response to nanocarriers encapsulating TLR 7 agonist. Definitely, the development of more efficient drug delivery systems encapsulating TLR agonists could contribute to vaccine-based cancer immunotherapy.

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1. Introduction

1.1 Introduction to cancer immunotherapy

Cancer immunotherapy has shifted the cancer treatment paradigm from directly killing tumor cells to the activation of host immune system to induce potent antitumor immune response with the potential for fewer off-target effects [3]. Agents used in cancer immunotherapeutics, activating host immune cells to induce tumor cell death, have demonstrated promising results in clinical applications [4, 5]. Nevertheless, antitumor efficacy of cancer immunotherapy is often hindered by delivery barriers as well as immunosuppressive tumor microenvironment [3, 6]. Thus, both the enhancement of delivery efficacy and modulation of tumor microenvironment are required for improving clinical outcome of cancer immunotherapeutics.

Cancer immunotherapies approved by US Food and Drug Administration (FDA) mainly involve chimeric antigen receptor (CAR) T cells, cytokines for lymphocyte activation, checkpoint inhibitors, and cancer vaccines [3]. The CAR T cell approach is a highly personalized anticancer therapy. Briefly, T cells collected from tumor-bearing patients are genetically engineered to express CARs specific for antigens displayed on cancer cells. Upon re-administration of engineered T cells to the same patient, CAR T cells are able to recognize the targeted tumor antigen and kill tumor cells [7, 8].

Cytokines used in cancer immunotherapy are injected directly to activate immune response [3]. Lymphocyte promoting cytokines involve three main types, interferons, interleukins, and granulocyte–macrophage colony-stimulating factor (GM-CSF) [9]. Immune checkpoint inhibitors are considered as one of the most promising immunotherapies. Two most widely used checkpoint inhibition strategies are CTLA4 blockade and PD-1/PD-L1 inhibition, which induce T cell-mediated tumor cell death through the removal of co-inhibitory signals preventing anticancer T cell function [10]. Cancer vaccine approaches for immunotherapy are described in more detail in the next section.

1.2. Cancer vaccine principles and hurdles

Cancer vaccines are recognized as one of the most promising strategies in the field of immunotherapy, with their unique capability of targeting immune cells and co-delivering antigen and adjuvants to induce antitumor immune response [11]. To date, remarkable progress has been made in the production of cancer vaccine for clinical cancer treatment. The autologous tumor-cell vaccine has been demonstrated to induce immune response in melanoma-bearing patients [12]. In addition, cancer vaccines derived from whole allogeneic cells also have many advantages. Melacine, an allogeneic melanoma tumor cell lysate vaccine, showed survival benefits in clinical trials [13].

An effective cancer vaccine consists of three essential components: antigen, adjuvant, and delivery system [14]. Antigens are molecules, such as peptides, proteins or lipids, which are detected by the immune system and elicit an antigen-specific immune response. Adjuvants act as stimulants of immune system and are able to influence the profile of induced immune response, while the delivery system is a platform that enables optimal co-delivery of antigen and adjuvant for the effective activation of both innate and adaptive immune systems [14]. It has been demonstrated that the efficacy of a cancer vaccine can be significantly improved by developing delivery tools capable of protecting and delivering antigens and adjuvants simultaneously [15].

One of the main obstacles of cancer vaccine development is the selection of appropriate antigens [14]. In order for potent antitumor immune response, sufficient tumor associated antigens (TAAs) are required for the activation of dendritic cells (DCs), leading to T cell proliferation and differentiation into effector cells [16]. TAAs can be of several types. Two main categories are shared tumor antigens and unique tumor antigens. Shared tumor antigens are overexpressed by multiple types of tumor, whose expression on healthy tissues is either absent or quantitatively different, such as melanoma-assiciated antigen (MAGE) and Cancer/Testis Antigen 1B (CTAG1B) [17, 18]. The second category comprises antigens, which are expressed uniquely by

individual tumors and correspond to new epitopes [19]. However, most TAAs have several drawbacks when tested *in vivo*, including risk of low immunogenicity, problems of antigen processing and presentation, and slow and weak antigen-specific T cell response [14]. Apart from problems relevant to tumor antigens, additional hurdles of the application of cancer vaccine have to be overcome. Barriers contribute to the ineffectiveness of cancer vaccines also include T cell exhaustion by immune checkpoint molecules, such as PD-1/PD-L1 and CTLA 4, expression of immunosuppressive molecules, such as interleukin-10, and MHC class I and T cell relevant tumor antigen downregulation by tumor cells [20-23]. Finally, selecting the optimal dose of cancer vaccines as well as the validation of evaluation methods of their effectiveness are both challenging [14]. Undoubtedly, well-designed studies are required for further improving the efficacy of cancer vaccines and broadening their therapeutic benefits in clinical application.

1.3. Toll-like receptor (TLR) signaling for DC activation

TLRs, sensors for pathogens, consist of an extracellular region, containing leucine-rich repeat motifs, and a cytoplasmic tail, which has a Toll/interleukin-1(IL-1) receptor (TIR) domain. One of the mechanisms by which the host immune system detects the invasion of pathogenic microbes is through TLRs [24]. Recognition of pathogen-associated molecular patterns (PAMPs) via TLRs activates DCs, causing initiation of adaptive immunity. TLR signaling in DCs leads to an increase in expression of MHC peptide ligands, upregulation of costimulatory molecules and secretion of immunomodulatory cytokines, critical for T cell expansion and differentiation into effector T cells [25]. There are twelve expressed TLR genes in mice and ten in humans. Each TLR is dedicated to the recognition of a distinct set of molecular patterns [26]. In humans, TLRs 1, 2, and 6, cell surface receptors, recognize lipids. TLRs 4 and 5 are also located on the cellular membrane of DCs, which recognize lipopolysaccharide (LPS) and flagellin, respectively. TLRs 3, 7, 8, and 9 are

endosomal nucleotide sensors. TLR 3 is activated by double-stranded RNA, while TLR 7 and TLR 8 are stimulated by single-stranded RNA, moreover, TLR 9 recognizes unmethylated CpG dinucleotides [26].

After ligand binding, TLRs signaling elicits a range of intracellular responses. TLRs 1, 2, 4, 5, 6, 7, 8, and 9 stimulate MyD88, while TLR 3 activates only TIR domain-containing adaptor protein inducing IFNβ (TRIF) signaling pathway [26]. The TRIF signaling stimulates interferon regulatory factor (IRF) to induce type I interferon gene expression, furthermore, myeloid differentiation primary-response gene 88 (MyD88) signaling activates mitogen-activated protein kinases (MAPKs), the transcription factor nuclear factor-kappa B (NF-κB), and members of IRF family, leading to the secretion of type I interferon and diverse pro-inflammatory cytokines, such as interleukin-12 and tumor necrosis factor alpha (TNF-α) [26].

1.4. TLR 7/8 agonists as immunostimulatory adjuvants of cancer vaccine

TLR agonists are one of the most commonly used vaccine adjuvants, which are co-administered with TAA for enhanced vaccine-based cancer immunotherapy. Specifically, synthetic small molecule imidazoquinolines, such as imiquimod (IMQ), resiquimod, and their derivatives, are effective TLR 7 or TLR 8 specific or TLR 7/8 bispecific agonists. They have demonstrated great potential as vaccine adjuvants, since they are able to improve the immunogenicity of TAA and enhance antigen-specific immune response [27].

IMQ, TLR 7 specific agonist, was approved by FDA for the treatment of basal cell carcinoma in 1997 [28]. Both IMQ and resiquimod are cytokine inducers, capable of activating plasmacytoid DCs, the primary interferon-producing cells, to stimulate interferon-α secretion [29]. They also induce the production of interleukin-12 and TNF-α, and enhance costimulatory marker expression [29, 30]. These secreted cytokines are able to further contribute to T cell proliferation and differentiation into $CD8^+$ cytotoxic T lymphocyte (CTL) and induce T cell-mediated immunity [31]. The chemical structure

of IMQ and resiquimod are shown in **Figure 1**.

(A) **(B)**

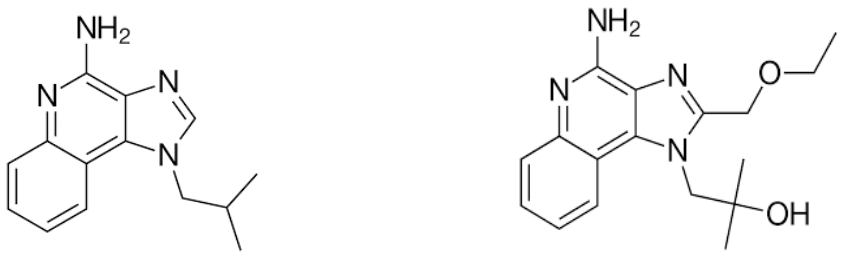


Figure 1. Chemical structure of IMQ (A) and resiquimod (B)

1.5. Biodegradable nanoparticles (NPs) as vaccine delivery system

To date, numerous studies have shown the success of biodegradable NPs as vaccine delivery vehicles for cancer immunotherapy [34, 35]. In order to fabricate effective vaccine delivery systems, extensive research has been conducted to evaluate the antigen uptake, processing into peptides and presentation via MHC class I by DCs, and DC migration to lymph nodes and maturation. These are the key steps in triggering robust antigen-specific immune response [36].

Uptake of biodegradable NPs by DCs depends on several physiochemical properties, including size, surface charge, shape, as well as hydrophobicity and hydrophilicity [14, 36]. Among these factors, size and surface charge of NPs play a crucial role in the internalization by DCs. It is reported that the optimal particle diameter for efficient DC uptake was $\leq$ 0.5 μm [37]. NPs within this size range might further trigger both humoral and cellular immune response. However, uptake of larger NPs by DCs could be improved by rendering the particle surface positive, which may arise from the greater affinity of positively charged molecules to the negatively charged cell membrane [14, 37]. Nevertheless, studies also suggested that the influence of particle surface charge on interaction with DCs is different for particles with diameter below 0.5 μm, and small-sized, anionic particles may have advantages over cationic particles, which may result in non-specific interaction with other cell types [37]. In addition,

intracellular trafficking studies indicated that some of cationic NPs could escape from lysosomes after being internalized and exhibit perinuclear localization, while the anionic and neutral NPs prefer to colocalize with lysosomes [38]. However, the interpretation of zeta potential effects on NP internalization by DCs can be complicated, and more thorough research is needed. Apart from general physiochemical characteristics, specific interactions arising from molecular functional groups of synthesized biodegradable NPs can be expected to exert an influence on NP interaction with DCs.

1.5.1 Poly(lactide-co-glycolide) (PLGA) NPs as delivery vehicles of TLR agonists

PLGA, a biocompatible and biodegradable FDA-approved copolymer, which could be hydrolyzed into lactic and glycolic acid monomers, has been extensively used in fabricating nanocarriers for drug delivery [39, 40]. PLGA-based NPs enable the encapsulation and delivery of a wide range of agents, including proteins, peptides, plasmid DNA, and hydrophilic or lipophilic drugs [41-44]. Moreover, the physiochemical properties of different PLGA-based NPs (size, morphology, zeta potential, drug release kinetics, degradation time), can be tuned by controlling molecular weight and copolymer ratio of PLGA, as well as parameters specific to the synthetic method [45, 46].

Poly (vinyl alcohol) (PVA) is a widely-used surfactant in formulating PLGA NPs with emulsion solvent evaporation method, which is able to generate relatively small particles with uniform size distribution [47]. The amount of residual PVA remaining associated with PLGA NPs has been shown to be a significant formulation parameter, which could affect the cellular uptake of NPs [48]. Furthermore, the introduction of didodecyldimethylammonium bromide (DMAB), a cationic surfactant, to modify PLGA-based NPs renders the particle surface charge positive and increases the stability of NPs [49, 50]. The properties and function of PLGA-based NPs could be further modulated by ligand attachment to the surface. For instance, ‘PEGlyation’,

attaching poly(ethylene glycol) (PEG) chains to the surface of PLGA NPs, is able to prolong the circulation half-life of NPs *in vivo* [51]. Furthermore, multiple ligands have been employed in the functionalization of PLGA-based NPs, including peptides, antibodies, proteins, glycolipids, and glycoproteins, to facilitate targeted drug delivery [46].

In addition to these promising results and applications, several studies have shown that PLGA-based NPs encapsulating TLR 7/8 agonists could achieve a sustained drug release profile and trigger DC activation and T cell response when co-administered with antigens [2, 34]. Furthermore, PLGA NPs containing poly(I:C) (TLR 3 agonist) or CpG oligodeoxynucleotide (ODN) (TLR 9 agonist) together with OVA were efficient at inducing antigen presentation by DCs and eliciting robust CTL responses [52]. Another study established that co-delivery of 7-acyl lipid A (TLR 4 agonist) and cancer-associated antigen by PLGA NPs could enhance DC maturation, trigger potent $CD8^+$ T cell response against cancer, and promote the secretion of pro-inflammatory cytokines [53]. These studies demonstrate the potential of PLGA-based NPs as competent vaccine delivery platforms.

1.5.2 Liposomes as delivery platforms of TLR agonists

It was discovered in the 1960s that hydration of dry lipid film in aqueous media could generate enclosed spherical vesicles or liposomes with lipid bilayers [54]. Generally, liposomes are composed of natural and/or synthetic lipids (phospho- and sphingo-lipids), which may also contain other bilayer components, such as cholesterol and hydrophilic polymer conjugated lipids [55]. What makes liposomes attractive delivery systems are their bilayered nature, which allows hydrophilic components to be either encapsulated into the inner aqueous core or adsorbed onto the liposomal surface, whereas the lipophilic part of the liposomes enables hydrophobic components to be incorporated within the membrane bilayers [56]. In addition, liposomal formulations can be optimized by altering their physicochemical characteristics,

including size, surface charge, surface hydration, and the fluidity of bilayer membrane, which exert a significant influence on the performance of liposomes *in vitro* and *in vivo* [55].

With more extensive studies and remarkable advancement in biomedical science, liposomes have been recognized as versatile biodegradable and biocompatible drug carriers to enhance the delivery efficiency of therapeutic agents [57]. Liu *et al.* developed a liposomal drug carrier through the attachment of substrate of endoprotease legumain, alanine–alanine–asparagine (AAN), to cell-penetrating peptides (TAT, trans-activating factor), to enhance the delivery efficiency of doxorubicin for cancer treatment and limit its toxic effects [58]. Moreover, Zhou *et al.* incorporated a mitochondrial targeting molecule onto the surface of paclitaxel liposomes, which showed potent anticancer efficacy for treating the drug-resistant lung cancer [59]. Also, Maruyama *et al*. designed the pendant type immunoliposome (34A-PEG-ILP), which was a long-circulating PEG-immunoliposome attached antibodies at the distal end of PEG chain [60]. Their study revealed that 34A-PEG-ILP exhibited much greater targetability to sites of action, solid tumor tissue and lung endothelial cells, compared with ordinary immunoliposomes.

In addition, recent studies have shown the potential of liposomes as vaccine delivery systems. Over the last few decades, the combination of liposomes with potent immunostimulatory adjuvants has arisen as a promising vaccine delivery platform for immunotherapy [61-63]. Poly(I:C) was effectively encapsulated into cationic liposomes with a high loading efficiency (> 96%), and poly(I:C)-loaded liposomes were capable of evoking a strong IgG1/IgG2a response [64]. Additionally, the co-encapsulation of OVA and CpG or PAM_3CSK_4 (TLR 2 ligand) in cationic liposomes was able to evoke DC activation, induce the production of IgG 2a and IgG1antibodies, trigger a robust cellular immune response, and promote the secretion of interferon-γ [65]. It was also reported that a nanoliposome delivery system that co-localized IMQ as well as GLA (TLR 4 agonist) could enhance Th1 adaptive immune responses and facilitate the production of interferon-γ [66]. Furthermore, another study demonstrated that liposomes

containing 3M-019, a new TLR 7 ligand, could induce IgG2a and IgG2b antibody response and trigger strong cellular immune response, leading to more powerful immunity than either TLR 7 ligand or liposomes alone [67].

Even though numerous studies have examined the influence of different antigens and adjuvants on inducing immune cell activation and immune response, further research is needed to dissect the influence of delivery systems on the efficacy of cancer vaccine. Furthermore, although both PLGA NPs and liposomes have been widely used as drug delivery platforms, the comparison of PLGA NPs with liposomes as delivery vehicles for cancer vaccine adjuvants may contribute to the discovery of more efficient drug carriers as vaccine delivery systems for enhanced cancer immunotherapy. In the present study, we fabricated cationic and anionic PLGA NPs as well as liposomes as four types of drug carriers, encapsulating the same immunostimulatory adjuvant, IMQ. Also, these nanoformulations were co-administered with the same model antigen, ovalbumin (OVA), to carry out *in vitro* studies in order to evaluate their influence on DC activation and antigen presentation.

1.6. Specific Aims

Specific aims of this study are as follows:

Specific aim 1. Development of cationic and anionic PLGA NPs as well as liposomes as drug carriers for TLR 7 agonist delivery.

Specific aim 2. *In vitro* evaluation of four types of nanocarriers on DC activation and antigen presentation.

2. Materials and Methods

2.1. Materials

PLGA (50:50 lactide-glycolide ratio; 0.55-0.75 dl/g inherent viscosity) was purchased from Lactel (Birmingham, AL). DMAB, IMQ, PVA, ammonium acetate and OVA from chicken egg white were purchased from Sigma-Aldrich (St Louis, MO). Chloroform and acetonitrile were purchased from Fisher Scientific (Rockford, IL). Fluorophore-labeled monoclonal antibodies were purchased from Biolegend (San Diego, CA) (CD80, CD86, CD11c, CD40, (I-A/I-E (MHC II)). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG), cholesterol, 18:0 PC 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). All cell culture media and buffers (including phosphate buffered saline) were purchased from Life Technologies (Carlsbad, CA) or Corning (Tewksbury MA).

Animals and cell lines

C57BL/6 mice (6-8 weeks, female) were purchased from Charles River (Wilmington, MA). Mice were housed under specific pathogen free (SPF) facilities in Research Animal Resources at the University of Minnesota. All animal experiments were performed according to the protocols approved by Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota. DC 2.4 cell line was provided by Professor Chun Wang, University of Minnesota.

2.2. Fabrication of IMQ loaded PLGA NPs

IMQ loaded PLGA NPs were prepared using the water-in-oil-in-water (w/o/w) double-emulsion solvent evaporation technique [2]. The aqueous phase of primary w/o

emulsion was prepared by dissolving 2.5 mg of sodium bicarbonate in 500 µl of 1% w/v PVA in endotoxin-free distilled water (D.I. water). This aqueous phase was then added to the oil phase, which comprised 30 mg PLGA and 1.5 mg IMQ dissolved in 2 ml chloroform. The mixture was sonicated using a probe sonicator (Sonicator XL, Misonix, Melville, NY) for 1 min to generate w/o emulsion. The primary emulsion was then transferred to 8 ml of 2% w/v PVA solution and sonicated for 5 min to generate the secondary w/o/w emulsion. The final emulsion was stirred at room temperature for ~18 h, followed by 1 h of vacuum in a desiccator to evaporate chloroform. NPs were separated and washed by ultracentrifugation (Optima XPN-80 Ultracentrifuge, Beckman Coulter Inc., Fullerton, CA) (35,000 RPM, 35 min) and then resuspended in D.I. water. This procedure was repeated twice to remove unencapsulated drug and residual PVA. After final wash, NPs were dispersed in D.I. water containing 20 mg sucrose (cryoprotectant). PLGA NPs were then lyophilized (Labconco FreeZone 4.5, Kansas City, MO) and stored at -20 °C until further use.

2.3. Fabrication of IMQ loaded DMAB-PLGA NPs

DMAB modified IMQ loaded PLGA NPs were formulated using the aforementioned double-emulsion solvent evaporation technique with modifications. The aqueous phase of primary w/o emulsion was prepared by dissolving 2.5 mg of sodium bicarbonate in 500 µl of 1% w/v PVA in D.I. water. This aqueous phase was then added to the oil phase, which comprised 30 mg PLGA and 1.5 mg IMQ dissolved in 2 ml chloroform. The mixture was sonicated using probe sonicator (Sonicator XL, Misonix, Melville, NY) for 1 min to generate w/o emulsion. The primary emulsion was then transferred to 8 ml of 2% w/v PVA solution and sonicated for 5 min to generate the secondary w/o/w emulsion. The final emulsion was stirred in room temperature for ~18 h, followed by 1 h of vacuum in a desiccator to evaporate chloroform. NPs were separated and washed by ultracentrifugation (Optima XPN-80 Ultracentrifuge, Beckman Coulter Inc., Fullerton, CA) (35,000 RPM, 35 min) and

then resuspended in D.I. water. This procedure was repeated once to remove unencapsulated drug and residual PVA. Modification with DMAB was performed by resuspending NP pellets after the third centrifugation step in 1% w/v aqueous DMAB solution, followed by stirring overnight at room temperature. To remove unbound DMAB, NPs were then washed twice by ultracentrifugation and dispersed in D.I. water containing 20 mg sucrose (cryoprotectant). DMAB modified PLGA NPs were then lyophilized and stored at -20°C until further use.

2.4. Fabrication of IMQ loaded DSPC-liposomes

DSPC, cholesterol, and DSPE-PEG were dissolved in chloroform at a mole ratio of (65:31:4.5). The solvent was evaporated under vacuum overnight to generate lipid films. IMQ acetate buffer solution was prepared by dissolving 3 mg IMQ in 2.5 ml acetate buffer (pH 3.3). The dried lipid film was hydrated with IMQ acetate buffer solution at 60 °C for 1 h with intermittent vortex. The lipid suspension then underwent water bath sonication to generate liposomes dispersion (until the suspension changes from milky to nearly clear in appearance). Liposomes were then separated from free drug via size exclusion chromatography with a disposable PD-10 column (GE Healthcare, Pataskala, OH), and the buffer was exchanged for PBS (pH 7.4). The liposomes obtained were stored at 4 °C prior to use.

2.5. Fabrication of IMQ loaded DOTAP liposomes

DOTAP and cholesterol were dissolved in chloroform at a molar ratio of 1:1. The solvent was evaporated under vacuum overnight to generate lipid films. IMQ acetate buffer solution was prepared by dissolving 3 mg IMQ in 2.5 ml acetate buffer (pH 3.3). The dried lipid film was hydrated with IMQ acetate buffer solution at 60 °C for 1 h with intermittent vortex. The lipid suspension then underwent water bath sonication to generate liposomes dispersion (until the suspension changes from milky

to nearly clear in appearance). Liposomes were then separated from free drug via size exclusion chromatography with a disposable PD-10 column (GE Healthcare, Pataskala, OH), and the buffer was exchanged for PBS (pH 7.4). The liposomes obtained were stored at 4 °C prior to use.

2.6. Physiochemical characterization of NPs and liposomes

Size and zeta potential of NPs and liposomes were determined by dynamic light scattering (DLS) (Delsa™ Nano C, Beckman Coulter Inc.). For PLGA NPs and DMAB modified PLGA NPs, ~1 mg of NPs was dispersed in D.I. water and sonicated before measurements. For DSPC liposomes and DOTAP liposomes, 100 μl of liposomes dispersion was diluted in D.I. water before measurements. The amount of IMQ encapsulated into the NPs or liposomes was quantified using Ultraviolet (UV) analysis. Lyophilized NPs or liposomes were first dispersed in methanol at a concentration of 1 mg/ml and incubated overnight in room temperature. The dispersion was centrifuged at 13,000 RPM for 15 min and 100 μl of the supernatant was collected. The supernatant was then evaporated and redissolved in acetate buffer (pH 3.3) for UV analysis at a detection wavelength of 319 nm.

2.7. Culture of BMDCs

BMDCs were prepared using established protocols with modifications. Briefly, femurs and tibia were harvested from C57BL/6 mice, disinfected with 70% ethanol, and rinsed with PBS (pH 7.4). Bone marrow precursor cells were collected by flushing with PBS using a 27-gauge needle (Medtronic, Minneapolis, MN). The flushed bone marrow was filtered with a 70-μm nylon mesh, and erythrocytes were removed using lysis buffer (Pharm Lyse, BD Bioscience, San Jose, CA). After centrifugation, bone marrow precursor cells were seeded into petri dish and cultured with complete RPMI 1640 media supplemented with 20 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ) and

50 μM 2-mercaptoethanol (Sigma) at 37 °C with 5% CO_2 . BMDCS culture media was refreshed once on day 3, and it took 6 days to generate immature BMDCs.

2.8. Culture of DC 2.4 cells

DC 2.4 cells are immortalized murine DCs created by transducing bone marrow isolates of C57BL/6 mice with retrovirus vectors expressing murine GM-CSF and the *myc* and *raf* oncogenes. DC 2.4 cells between passages 7-10 were used for the study. Cells were cultured using established protocols with modifications [68]. Briefly, DC 2.4 cells were cultured in complete RPMI 1640 media supplemented with 10mM HEPES Buffer Solution and 0.0054×2-mercaptoethanol (Sigma) at 37°C in a humidified atmosphere containing 5% CO2.

2.9. Cell Viability Assay

BMDCs or DC 2.4 cells (10,000 cells/well) were seeded in a 96-well cell culture plate. Following cell attachment, BMDCs or DC 2.4 cells were treated with free IMQ (0.625, 1.25, 2.5, 5, 10, 20μM), IMQ loaded PLGA NPs, DMAB modified PLGA NPs, DSPC liposomes and DOTAP liposomes and blank NPs or liposomes for 24 h. The amount of loaded NPs or liposomes added was based on the drug loading to produce the same concentration as free IMQ. The concentration of blank NPs or liposomes was the same with the respective loaded ones. Treatments were incubated with BMDCs or DC 2.4 cells for 24h and cell viability was analyzed using MTS assay.

2.10. *In vitro* BMDCS and DC 2.4 cells activation

BMDCs or DC 2.4 cells (0.5×10^6/well) were seeded in a 24-well cell culture plate. Following cell attachment, BMDCs or DC 2.4 cells were treated with OVA (20μg) alone or OVA combined with free IMQ(100 ng/ml), IMQ loaded PLGA NPs,

DMAB modified PLGA NPs, DSPC liposomes and DOTAP liposomes and blank NPs or liposomes for 24 h. The amount of loaded NPs or liposomes added was based on the drug loading to produce the same concentration as free IMQ. The amount of blank NPs or liposomes was the same with the respective loaded ones. After 24 h, BMDCs or DC 2.4 cells were collected and analyzed for costimulatory molecules (CD40, CD86, and CD80) by flow cytometry.

2.11. *In vitro* antigen presentation by BMDCS or DC 2.4 cells

To assess *in vitro* antigen presentation, BMDCs or DC 2.4 cells (0.5×10^6/well) were seeded in a 24-well cell culture plate. Following cell attachment, BMDCs were treated with OVA (20 μg) alone or OVA combined with free IMQ (100 ng/ml), IMQ loaded PLGA NPs, DMAB modified PLGA NPs, DSPC liposomes and DOTAP liposomes and blanks NPs or liposomes for 24 h. The amount of loaded NPs or liposomes added was according to the drug loading to maintain the same concentration as free IMQ. The amount of blank NPs or liposomes was the same with the respective loaded ones. After 24 h, BMDCs or DC 2.4 cells were harvested and stained with anti-CD11c antibody and anti-$OVA_{257-264}$ (SIINFEKL) peptide bound to $H\text{-}2K^b$ antibody and analyzed by flow cytometry.

3. Results

3.1. Physiochemical Characterization of PLGA NPs and Liposomes

The hydrodynamic size of four types of nanoformulations was measured by DLS. PLGA NPs and DMAB- PLGA NPs had a similar average diameter (~250 nm), while the average diameter of DSPC liposomes and DOTAP liposomes was ~100 nm. PLGA NPs and DSPC liposomes were both negatively charged, with an average zeta potential of~ -25 mV, whereas DMAB-PLGA NPs and DOTAP liposomes were both

positively charged. The average zeta potential of DMAB-PLGA NPs was 23 ± 5.8 mV, however, DOTAP liposomes had a higher zeta potential of ~ 50 mV. The loading amount of IMQ in PLGA NPs and DMAB-PLGA NPs were similar at ~20 μg/mg, but the drug loading of both DSPC liposomes and DOTAP liposomes was smaller at ~10 μg/mg. (Table 1)

Table 1: Physicochemical characterization of NPs and liposomes

	PLGA NPs	DMAB-PLGA NPs	DSPC liposomes	DOTAP liposomes
Size (nm)	249 ± 36.5	274 ± 18.1	98 ± 11.4	100 ± 7.7
Zeta potential(mV)	-24 ± 11.2	23 ± 5.8	-26 ± 2.5	50 ± 25.4
PDI	0.2 ± 0.07	0.2 ± 0.08	0.2 ± 0.01	0.2 ± 0.07
Loading amount of IMQ (μg/mg NP or liposomes)	20.6 ± 5.0	23.8 ± 4.2	9.5 ± 1.2	8.75 ± 2.54

3.2. Cell viability assays

Cytotoxicity was evaluated in BMDCs (primary cells) and DC 2.4 (immortalized cells) by MTS assay. Nine treatments with concentrations (0.625, 1.25, 2.5, 5, 10, 20μM) were used in this study, including free IMQ, loaded PLGA NPs, loaded PLGA-DMAB NPs, loaded DSPC liposomes, loaded DOTAP liposomes, blank PLGA NPs, blank PLGA-DMAB NPs, blank DSPC liposomes, and blank DOTAP liposomes. Results indicated that free IMQ promoted the growth of BMDCs at low concentrations, but showed cytotoxicity at concentrations higher than 10 μM. Furthermore, both loaded PLGA NPs and DSPC liposomes, as well as their blank formulations have negligible cytotoxicity against primary cells. Nevertheless, both loaded DMAB-PLGA NPs and DOTAP liposomes, as well as their blank formulations showed cytotoxicity to primary cells at concentrations higher than 1.25 μM and 2.5 μM, respectively.

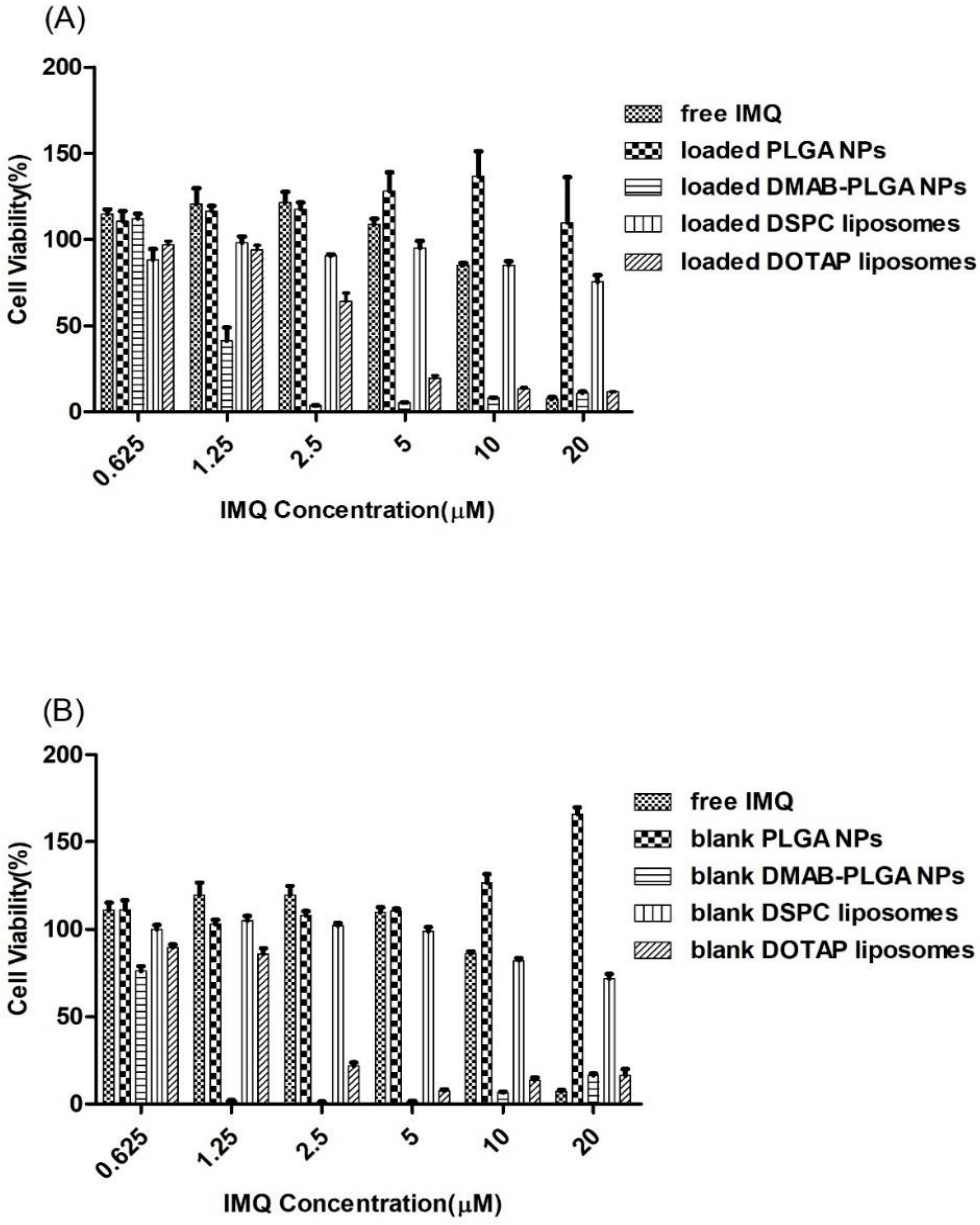


Figure 2. Cell viability of BMDCs

(A) Cell viability of BMDCs on treatments with free IMQ and IMQ loaded NPs and liposomes. Results are reported as mean ± SD, n=5. **(B)** Cell viability of BMDCs on treatments with free IMQ and blank NPs and liposomes. Results are reported as mean ± SD, n=5.

With regards to DC 2.4 cells, free IMQ also induced cell growth at concentrations lower than 5 μM, but showed cytotoxicity at concentrations higher than 10 μM.

Similar to primary cells, both loaded PLGA NPs and DSPC liposomes as well as their blank formulations show negligible cell-killing effects against immortalized cells. However, both loaded and blank DMAB-PLGA NPs showed cytotoxicity to DC 2.4 at concentrations greater than 2.5 μM. Also, both loaded DOTAP liposomes and its blank counterparts showed cytotoxicity at concentrations greater than 5 μM.

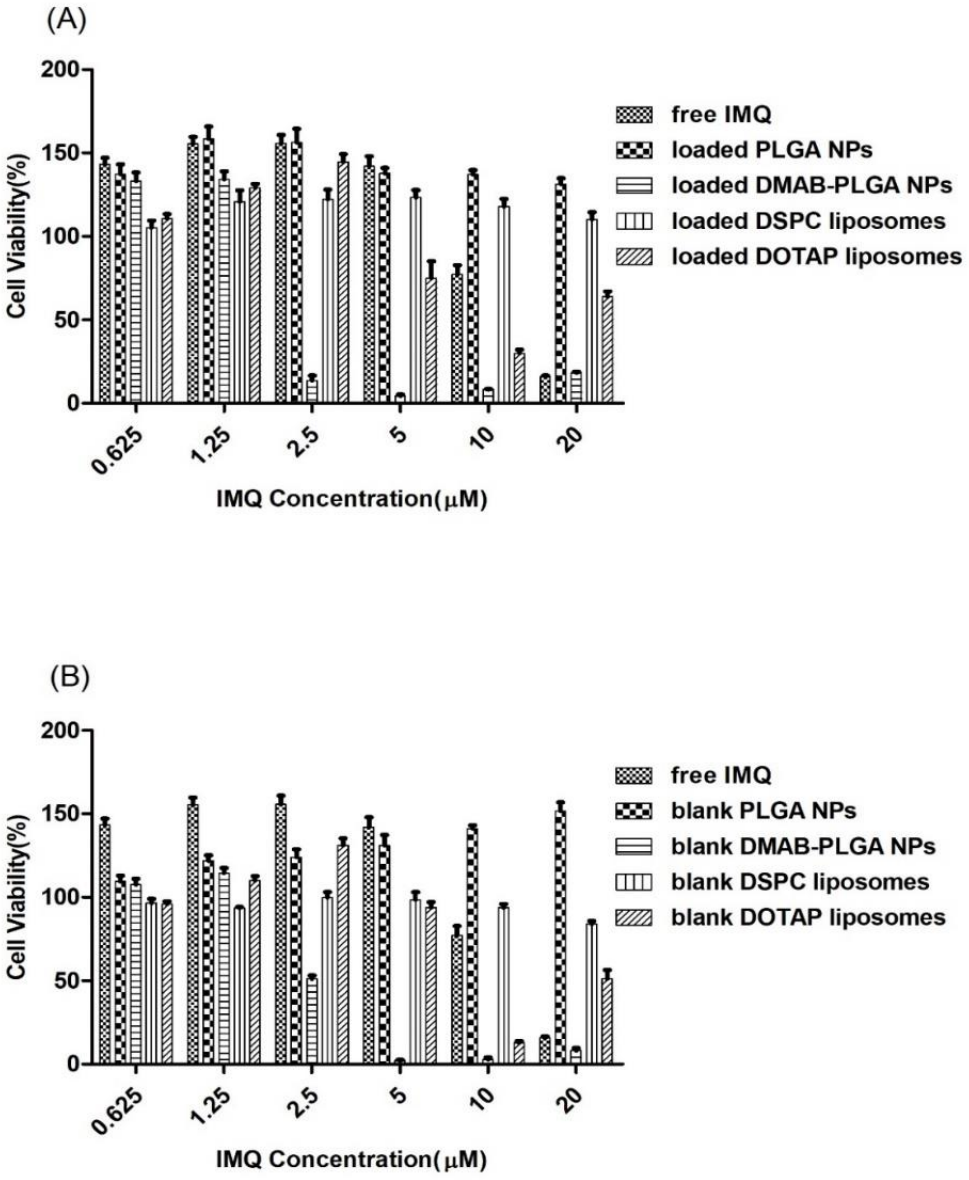


Figure 3. Cell viability of DC 2.4

(A) Cell viability of DC 2.4 on treatments with free IMQ and IMQ loaded NPs and liposomes. Results are reported as mean ± SD, n=5. **(B)** Cell viability of DC 2.4 on

treatments with free IMQ and blank NPs and liposomes. Results are reported as mean ± SD, n=5.

3.3. *In vitro* BMDCs activation

Here, we evaluated the expression of costimulatory molecules (CD40, CD80 and CD 86) to examine the extent of BMDC activation. As a model antigen, OVA was treated alone or co-administered with nine treatments, free IMQ, loaded PLGA NPs, loaded PLGA-DMAB NPs, loaded DSPC liposomes, loaded DOTAP liposomes, blank PLGA NPs, blank PLGA-DMAB NPs, blank DSPC liposomes, and blank DOTAP liposomes. As is indicated, OVA alone had a smaller effect on the expression of CD40 compared with OVA+free IMQ, OVA+loaded PLGA NPs, OVA+loaded DMAB-PLGA NPs and OVA+loaded DOTAP liposomes. CD 40 expression increased from 25% for OVA alone treatment to 50% for OVA+DOTAP liposomes treatment, 70% for OVA+DMAB-PLGA NPs, 76% for OVA+free IMQ treatment, and 92% for OVA+PLGA NPs treatment. However, CD 40 expression in all blank formulations and loaded DSPC liposomes with OVA was similar to OVA alone group.

In addition, CD80 expression by BMDCs was likely in treatments with OVA alone, OVA+free IMQ, OVA+blank PLGA NPs, OVA+blank/loaded DMAB-PLGA NPs, OVA+blank/loaded DSPC liposomes, and OVA+blank/loaded DOTAP liposomes. However, OVA+loaded PLGA NPs treatment increased the frequency of BMDC expressing CD80 to 32%, whereas OVA alone treatment only resulted in 12% of CD80 expression. Furthermore, OVA+free IMQ treatment induced slightly higher CD86 expression of BMDCs in comparison with OVA alone treatment as well as all blank formulations together with OVA (5%~10%). OVA+loaded PLGA NPs treatment led to the highest BMDC expression of CD86 (26%), whereas OVA+DMAB-PLGA NPs treatment caused the lowest CD86 expression, 7%. However, the frequency of CD86 expression in treatments of OVA alone, OVA+free IMQ, OVA+loaded DSPC liposomes and OVA+loaded DOTAP liposomes were all within the range from 15%

to 20%.

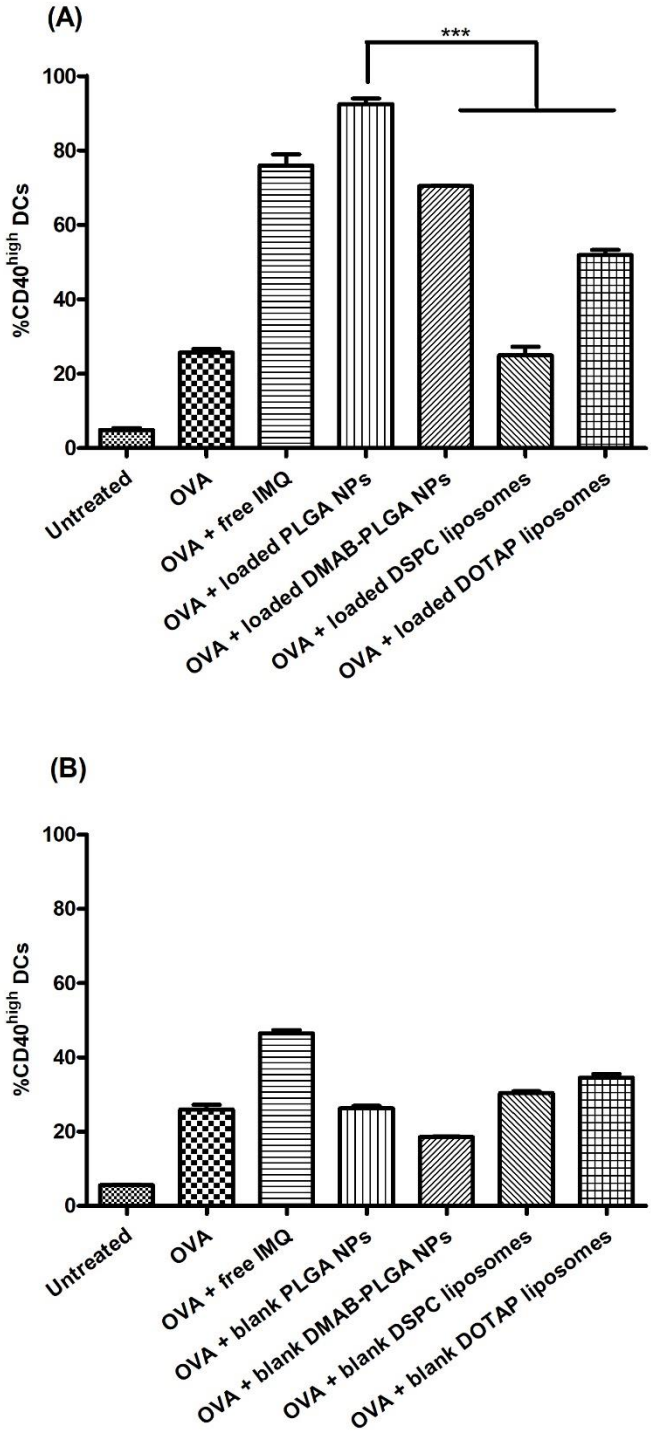


Figure 4. Expression of CD 40 on BMDCs

(A) Flow cytometry analysis of CD40 expression on BMDCs after 24 h incubation with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are

reported as mean ± SD, n=3, ***p<0.001, One-way ANOVA. **(B)** CD40 expression on BMDCs after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are reported as mean ± SD, n=3.

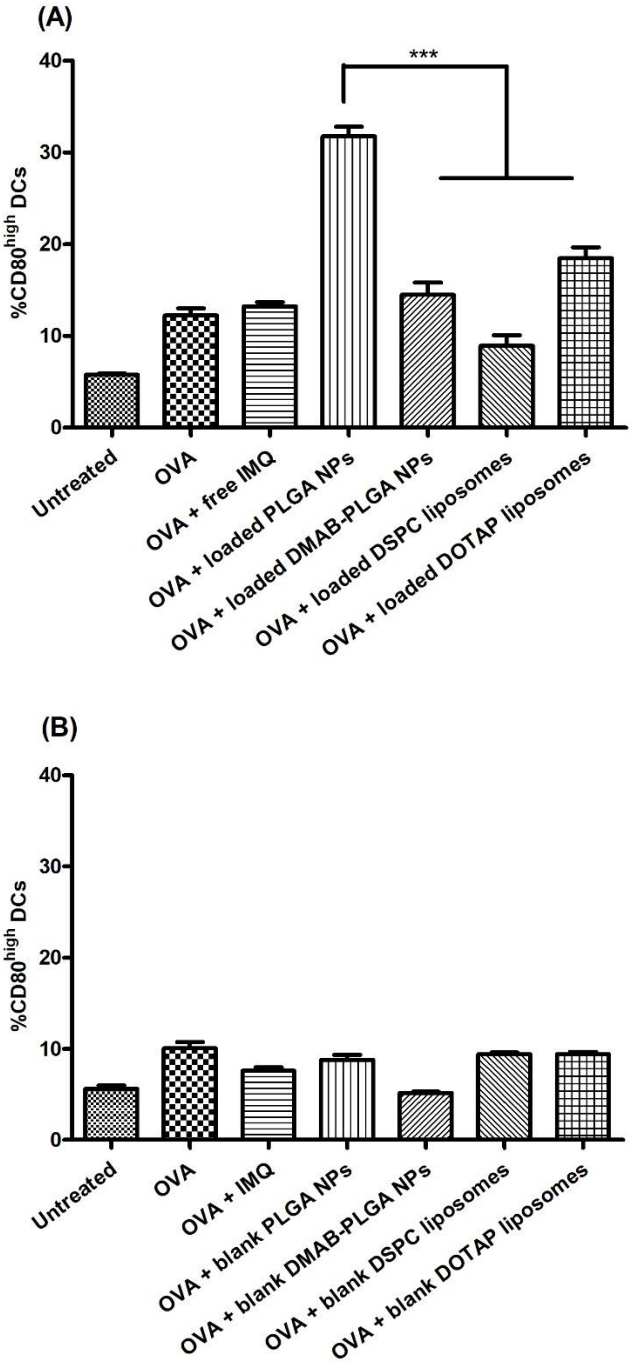


Figure 5. Expression of CD 80 on BMDCs

(A) Flow cytometry analysis of CD80 expression on BMDCs after 24 h incubation

with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are reported as mean ± SD, n=3, ***p<0.001, One-way ANOVA. **(B)** CD80 expression on BMDCs after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are reported as mean ± SD, n=3.

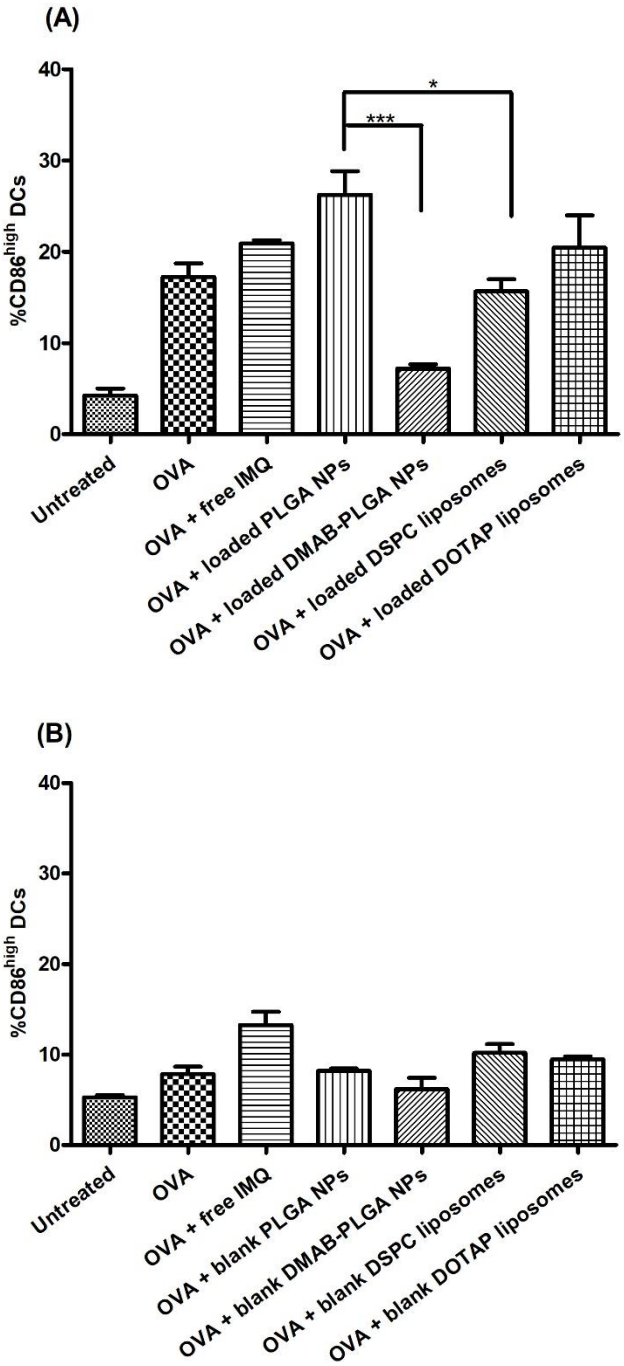


Figure 6. Expression of CD 86 on BMDCs

(A) Flow cytometry analysis of CD86 expression on BMDCs after 24 h incubation with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are reported as mean ± SD, n=3, *p<0.05, ***p<0.001, One-way ANOVA. **(B)** CD86 expression on BMDCs after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are reported as mean ± SD, n=3.

3.4. *In vitro* DC 2.4 activation

Apart from primary DCs, we also evaluated the influence of four types of nanocarriers together with their blank formulations on the activation of immortalized DCs. Treatments were the same as those treated BMDCs. As is shown, OVA+free IMQ treatment achieved the highest DC 2.4 expression of CD40 compared with all the other treatments. However, treatment of both OVA+loaded DMAB-PLGA NPs and its blank counterpart resulted in negligible CD40 expression by DC 2.4 cells. OVA alone treatment led to similar CD40 expression frequency (40%) in comparison with treatments of OVA+blank PLGA NPs, OVA+blank DSPC liposomes, and OVA+blank DOTAP liposomes, whereas OVA alone treatment caused slightly higher expression of CD40 compared with OVA+loaded PLGA NPs (23%), OVA+loaded DSPC liposomes (28%), and OVA+loaded DOTAP liposomes (30%). Additionally, OVA+loaded PLGA NPs treatment achieved the greatest expression frequency of CD80 (46%) among all the groups, while treatments of both OVA+loaded DMAB-PLGA NPs and its blank formulations caused no CD80 expression.

The frequency of DC 2.4 expressing CD80 were similar in treatments of OVA+free IMQ, OVA+loaded DOTAP liposomes and its blank counterparts, OVA+blank DSPC liposomes, which was greater than that of OVA+loaded DSPC liposomes (11%). Moreover, OVA+free IMQ obtained the highest expression level of CD86 among all treatments. OVA alone treatment achieved similar expression frequency of CD86 (5.8%) to that of treatments of all blank formulations co-administered with OVA. Nevertheless, CD86 expression by DC 2.4 cells with OVA

alone treatment was higher than that of treatments of all loaded formulations co-administered with OVA.

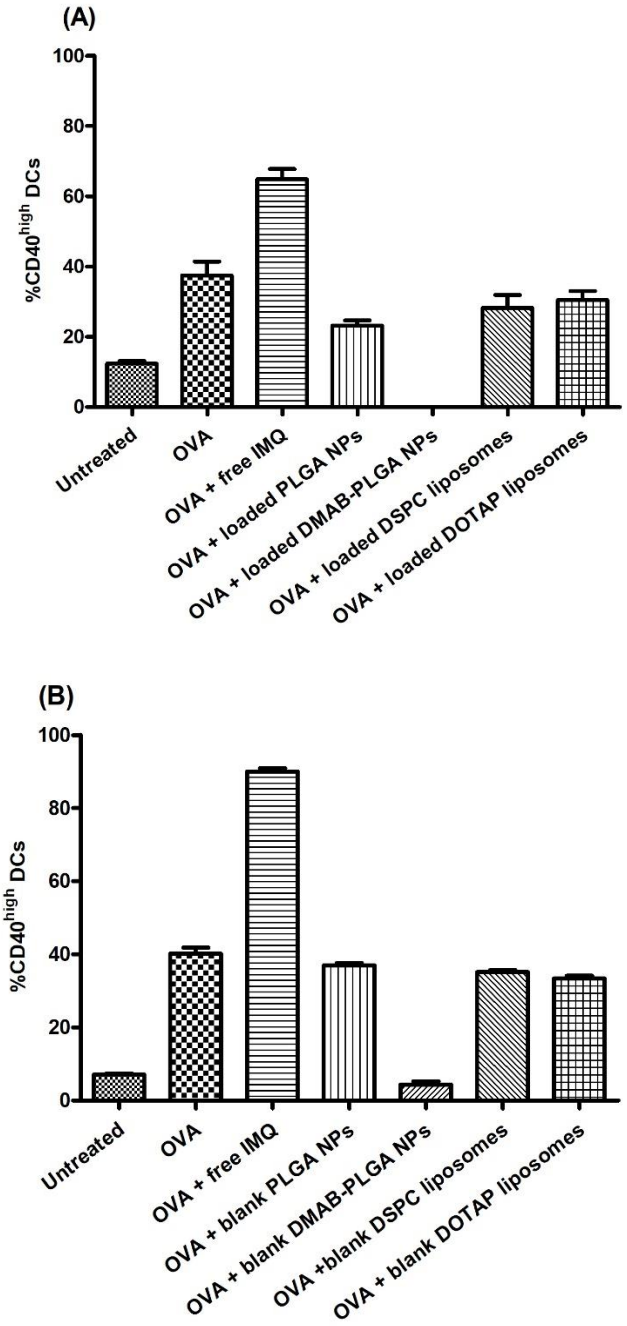


Figure 7. Expression of CD 40 on DC 2.4

(A) Flow cytometry analysis of CD40 expression on DC 2.4 after 24 h incubation with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are reported as mean ± SD, n=3. **(B)** CD40 expression on DC 2.4 after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are

reported as mean ± SD, n=3.

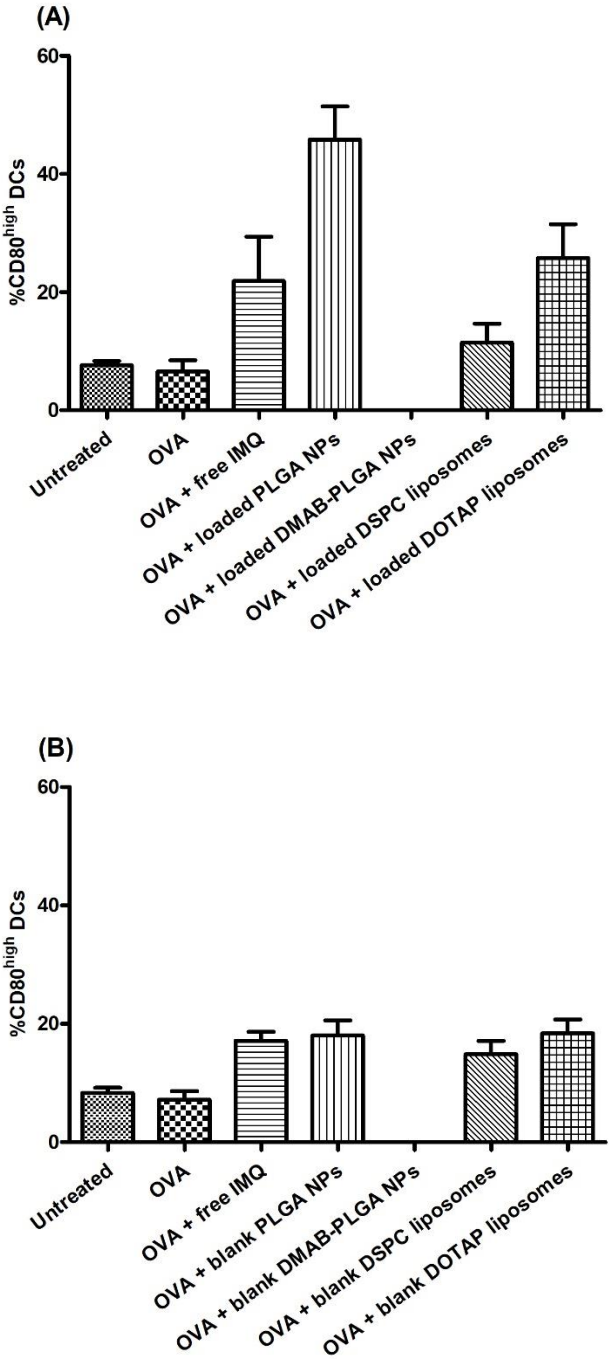


Figure 8. Expression of CD 80 on DC 2.4

(A) Flow cytometry analysis of CD80 expression on DC 2.4 after 24 h incubation with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are reported as mean ± SD, n=3. **(B)** CD80 expression on DC 2.4 after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are reported as mean ± SD, n=3.

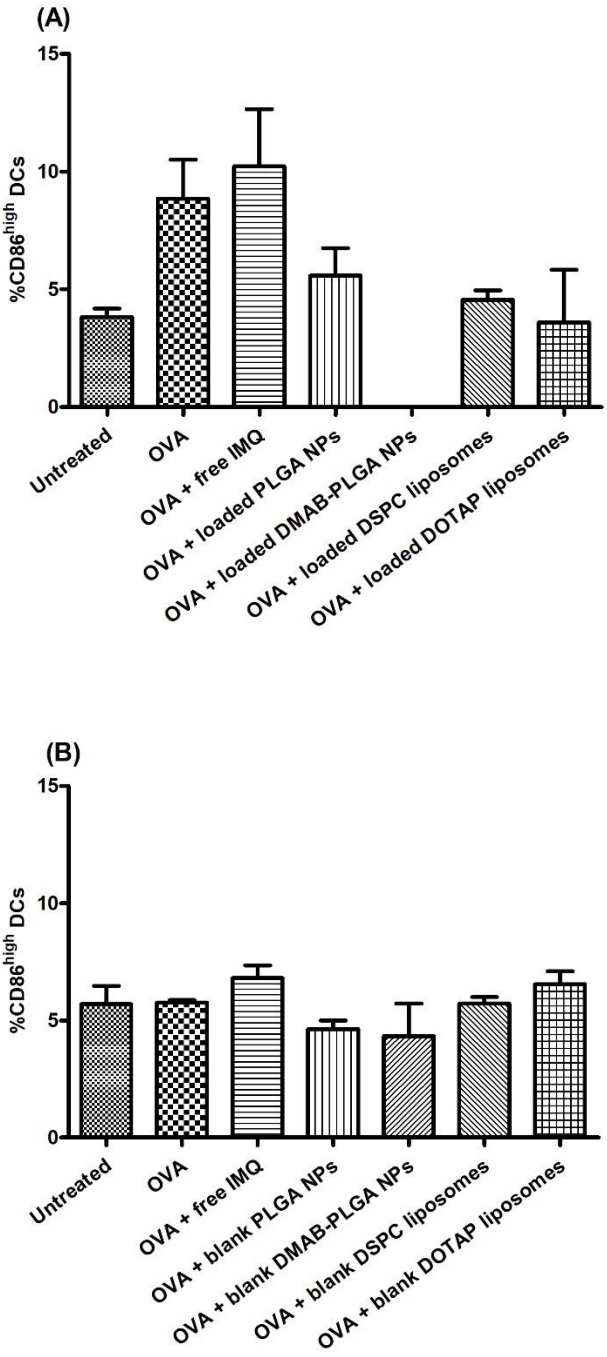
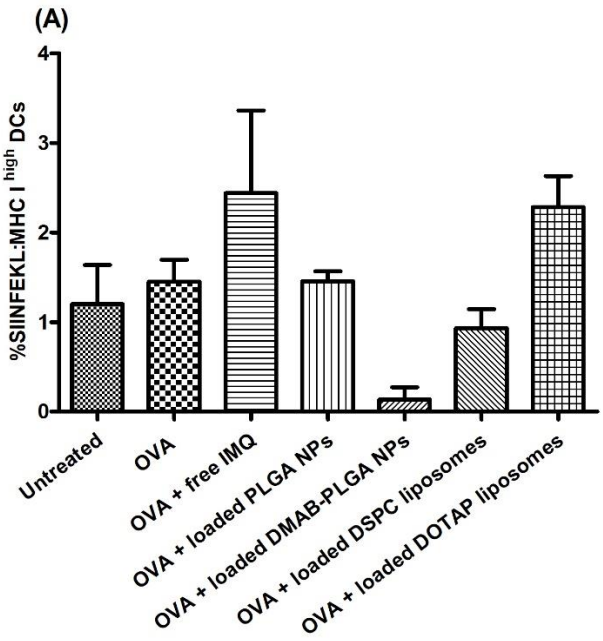


Figure 9. Expression of CD 86 on DC 2.4

(A) Flow cytometry analysis of CD86 expression on DC 2.4 after 24 h incubation with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are reported as mean ± SD, n=3. **(B)** CD86 expression on DC 2.4 after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are reported as mean ± SD, n=3.

3.5. *In vitro* antigen presentation by BMDCs

Apart from the expression of costimulatory molecules (CD40, CD80, CD86), we also examined another key feature of effective DC activation, antigen presentation via MHC I, which was measured through evaluating the expression of $OVA_{257-264}$(SIINFEKL) peptide:MHC I complex on BMDCs. As is indicated, OVA+free IMQ treatment obtained the greatest $OVA_{257-264}$(SIINFEKL) peptide:MHC I complex expression among all the treatments. However, both DMAB-PLGA NPs and its blank counterpart co-treated with OVA achieved negligible antigen presentation via MHC I. Additionally, the expression frequency of $OVA_{257-264}$(SIINFEKL) peptide:MHC I complex among treatments of OVA alone, both loaded PLGA NPs and its blank counterparts together with OVA, both loaded DSPC liposomes its blank counterparts together with OVA, and OVA+blank DOTAP liposomes, which was slightly lower than that of OVA+loaded DOTAP liposomes (2.3%).



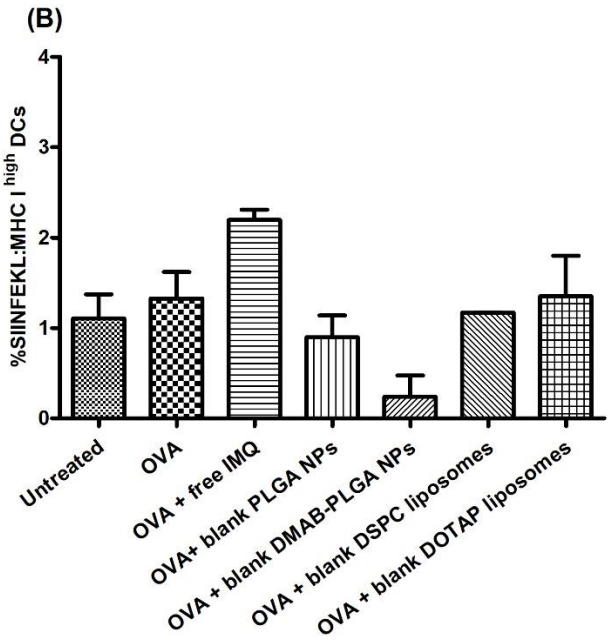


Figure 10. SIINFEKL: MHC I Expression of BMDCs

(A) Flow cytometry analysis of BMDCs expressing $OVA_{257-264}$ (SIINFEKL) peptide bound to H-$2K^b$, after 24 h incubation with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are reported as mean ± SD, n=3. **(B)** BMDCs expressing $OVA_{257-264}$ (SIINFEKL) peptide bound to H-$2K^b$, after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are reported as mean ± SD, n=3.

3.6. *In vitro* antigen presentation by DC 2.4

The extent of antigen expression via MHC I was also investigated in DC 2.4 cells. Similar to BMDCs, OVA+free IMQ treatment achieved the hightest $OVA_{257-264}$(SIINFEKL) peptide:MHC I complex expression among all the treatments. Also, both DMAB-PLGA NPs and its blank counterpart co-treated with OVA achieved no antigen presentation via MHC I. Furthermore, among all loaded formulations, OVA+DOTAP liposomes obtained the greatest expression frequency of $OVA_{257-264}$(SIINFEKL) peptide:MHC I complex (12%). Similarly, the expression level of OVA+blank DOTAP liposomes was the greatest among all blank formulations (7%).

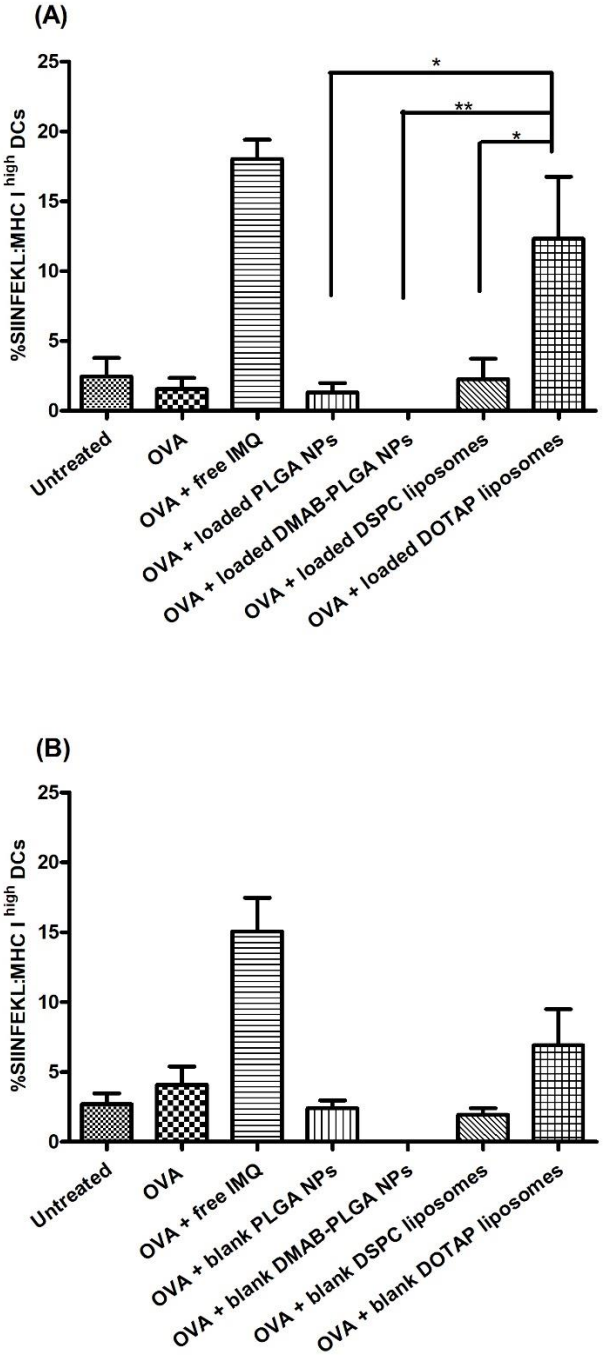


Figure 11. SIINFEKL: MHC I Expression of DC 2.4

(A) Flow cytometry analysis of DC 2.4 expressing $OVA_{257-264}$ (SIINFEKL) peptide bound to H-$2K^b$, after 24 h incubation with OVA alone, OVA combined with free IMQ, loaded NPs or liposomes. Results are reported as mean ± SD, n=3, *$p<0.05$, **$p<0.01$, One-way ANOVA. **(B)** DC 2.4 expressing $OVA_{257-264}$ (SIINFEKL) peptide bound to H-$2K^b$, after 24 h incubation with OVA alone, OVA combined with free IMQ, blank NPs or liposomes. Results are reported as mean ± SD, n=3.

4. Discussion

Immunotherapy for cancer treatment aims to activate the host immune system to induce potent anticancer immunity through the stimulation of T cells to recognize and eliminate cancer cells in an antigen-specific manner [2, 3]. Furthermore, TLR signaling in DCs could result in an increase in the expression of MHC peptide ligands, upregulation of costimulatory molecules and secretion of immunomodulatory cytokines, critical for T cell expansion and differentiation into CTL [25]. Current research has shown the progress in cancer immunotherapy with the development and application of cancer vaccine, which allows co-delivery of both TAA and immunostimulatory adjuvants [11, 12].

In particular, biodegradable and biocompatible PLGA-based NPs containing cancer-associated antigen and TLR agonists are efficient at promoting DC maturation and activation, inducing antigen presentation by DCs via MHC I, eliciting the secretion of pro-inflammatory cytokines, and triggering potent CTL response against cancer [52, 53]. In addition to PLGA-based NPs, bilayered liposomes, consisting of natural and/or synthetic lipids, are also recognized as attractive vaccine delivery systems, since the membrane fusion between liposomes and cells enables the efficient delivery of agents encapsulated within liposomes [69]. Similar to PLGA-based NPs, numerous studies have also demonstrated the potential of liposomes, especially cationic liposomes, in co-delivering tumor-specific antigens and immunostimulatory adjuvants to induce the production of IgG 2a and IgG1antibodies, elicit robust cellular immune response, and promote the secretion of IFN-γ [64, 65].

With these promising results, our aim focused on comparing the efficacy of PLGA NPs with liposomes as delivery platforms for vaccine adjuvant and studying the formulation effects on immune response to nanocarriers encapsulating TLR 7 agonist. In the present study, we fabricated both cationic and anionic PLGA NPs as well as liposomes as four types of delivery vehicles, encapsulating the same immunostimulatory adjuvant, IMQ, to activate primary DCs and immortalized DCs. Both PLGA NPs and DMAB-PLGA NPs had an average diameter of ~250 nm,

whereas the average particle size of DSPC liposomes and DOTAP liposomes were ~100 nm. Therefore, the diameters of all nanoformulations are within the size range preferential for DC internalization (<500 nm)， compared to PLGA macroparticles with an average size of ~1000nm [70]. In addition, the loading amount of IMQ in PLGA NPs and DMAB-PLGA NPs was similar, ~20 μg/mg, close to the loading efficiency of PLGA macroparticles [70]. Nevertheless, the IMQ content in two types of liposomes were both ~ 10μg/mg. The differences in drug loading efficiency among four types of nanoformulations might be due to the fact that double-emulsion evaporation method could lead to greater loading efficiency of imidazoquinoline derivatives in PLGA NPs [2]. Furthermore, it was previously reported that using thin-film hydration and passive loading method to encapsulate imidazoquinolines, resiquimod, resulted in relatively low encapsulation efficiency (final weight loading of ~1.1%) [71]. As such, further studies are required to improve the loading efficiency of IMQ in liposomal formulations and achieve similar loading efficiency in all types of drug carriers for a comparable evaluation *in vitro* or *in vivo*.

Cell viability assay was conducted to examine the cytotoxicity of four types of nanoformulations before *in vitro* DC activation and antigen presentation assay. As is indicated in our results, IMQ loaded PLGA NPs as well as their blank formulations had negligible cytotoxicity on both BMDCs and DC 2.4. However, IMQ loaded DSPC liposomes as well as their blank formulations showed cytotoxicity on BMDCs at concentrations greater than 10μM, which on the other hand, exerted negligible toxic effects on DC 2.4. IMQ loaded DMAB-PLGA NPs and their blank counterpart showed obvious cytotoxicity on BMDCs even when concentration was higher than 1.25 μM, on DC 2.4 when concentration was greater than 2.5 μM. Additionally, both blank DOTAP liposomes and their loaded counterparts indicated cell-killing effect on BMDCs at concentrations greater than 1.25 μM, which, nevertheless, showed cell-killing effect on DC 2.4 cells at concentrations higher than 10 μM.

These results demonstrated better tolerance of immortalized DCs than primary DCs. Additionally, among four types of nanoformulations, PLGA NPs were the most

biocompatible nanocarriers with negligible cytotoxicity, which has been revealed in the previous study [2]. In contrast, both DMAB-PLGA NPs and DOTAP liposomes exhibited obvious cytotoxic effects even at relatively low concentrations. One possible explanation was that cationic NPs were often cytotoxic due to their attractive interaction with negatively charged cell membranes and disruption of membrane integrity [72]. Furthermore, two main components of these two nanoformulations, DMAB and DOTAP, had nonnegligible cytotoxicity, which have also been indicated in previous studies [73, 74]. Improvements could be made through altering the ratio of DMAB or DOTAP in each cationic nanoformulation or synthesize lipid-biopolymer hybrid NPs in order to reduce the cytotoxicity [35]. It is also reported that adsorption of cationic polymer carboxymethyl trimethyl chitosan (CM-TMC) to PLGA NPs could not only render the particle surface charge positive, but also show no significant cytotoxicity [75].

In the above results, IMQ loaded PLGA NPs + OVA treatment induced the greatest costimulatory molecules (CD40, CD80, CD86) expression by BMDCs compared with other treatments, which further confirmed the superiority of PLGA NPs as vaccine adjuvants delivery platforms [2, 34]. On the other hand, with respect to the DC 2.4 cells, OVA + free IMQ treatment were able to induce the highest expression of CD86 and CD40, albeit OVA + loaded PLGA NPs resulted in the greatest expression of CD80 among all the treatments. One potential explanation was that since the incubation time of treatments was 24h, IMQ encapsulated within the nanoformulations might not release completely. Thus, *in vitro* release study was required to evaluate the drug release profile of these nanocarriers. However, OVA + DMAB-PLGA NPs led to negligible expression of costimulatory molecules by DC 2.4. We hypothesized that strong cytotoxicity of DMAB-PLGA NPs might impair the DCs' capability of expressing costimulatory molecules.

Regarding antigen presentation by BMDCs or DC 2.4 cells, our results indicated that of all nanocarriers, DOTAP liposomes could induce the highest expression of $OVA_{257-264}$(SIINFEKL) peptide:MHC I complex, whereas the antigen expression

frequency in OVA+free IMQ treatment was greater than control and all the other formulation treatments. One possible explanation was that cationic DOTAP liposomes were more effective in promoting the antigen presentation by DCs via MHC I, which was critical in T cell activation and differentiation into functional CTLs. Also, Chen *et al.* reported that E7 peptide formulated with DOTAP could induce migration of activated DCs to the draining lymph node and generate robust antigen-specific $CD8^+$ T lymphocyte responses [76]. Furthermore, the superiority of OVA + free IMQ treatment to nanoformulation treatments in eliciting antigen presentation by DCs might also be partially due to the incomplete release of IMQ encapsulated within these nanocarriers. Also, we hypothesized that the strong cytotoxicity of DMAB-PLGA NPs might negatively affect the ability of DCs to process and present antigen peptide via MHC I. Definitely, more research is required to investigate the mechanism of formulation effects, particularly size and surface charge of nanoformulations, on immune response to different nanocarriers encapsulating TLR 7 agonist.

5. Conclusion

As one of the most promising strategies in cancer immunotherapy, cancer vaccines possess the unique ability to target host immune systems and co-deliver antigen and immunostimulatory adjuvants to induce antitumor immune response [11]. The stimulation of T cell-mediated immunity through inducing the activation of DCs, the critical APCs, via TLR signaling have gained worldwide interests [25, 29-31]. Here, we fabricated both cationic and anionic PLGA NPs as well as liposomes as vaccine adjuvant delivery vehicles to encapsulate the FDA-approved TLR 7 agonist, IMQ, and carry out preliminary *in vitro* DC activation and antigen presentation studies to evaluate the formulation effects on immune response to nanocarriers encapsulating TLR 7 agonist. We successfully developed four types of nanoformulations, PLGA NPs and DSPC liposomes, which were negatively charged, and DMAB-PLGA NPs and DOTAP liposomes, which were positively charged. The diameters of all

nanoformulations are within the size range preferential for DC internalization. In addition, both PLGA NPs and DSPC liposomes showed negligible cytotoxicity on DCs, whereas both DMAB-PLGA NPs and DOTAP liposomes exhibited obvious cell-killing effects on DCs at low concentrations. Generally, anionic PLGA NPs were superior to other nanoformulations in eliciting the expression of costimulatory molecules by DCs, whereas cationic DOTAP liposomes were superior in inducing antigen presentation by DCs. Furthermore, DMAB-PLGA NPs resulted in negligible expression of costimulatory molecules by DC 2.4, which also caused almost no antigen expression by both BMDCs and DC 2.4. We expect these *in vitro* data can bring us one step further to understand the mechanism of formulation effects on immune response to different nanocarriers encapsulating TLR 7 agonist.

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